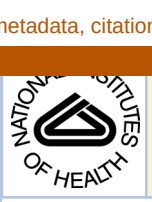

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Improved detection of latent *Mycobacterium tuberculosis* infection in HIV-1 seropositive individuals using cultured cellular assays

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Tuberculosis (TB), caused by the respiratory pathogen *Mycobacterium tuberculosis* (Mtb) is the leading cause of morbidity and mortality in patients coinfected with HIV-1. For the majority of immunocompetent individuals, Mtb replication is contained, and progression to active disease prevented by an orchestrated $CD4^+$ T-cell specific and Th1-dominant immune response [1]. These individuals are classified as latently infected with TB (LTBI) and have a 10% lifetime risk of developing TB [2]. The progressive and generalized $CD4^+$ T-cell depletion that occurs following HIV-1 infection increases the occurrence of TB disease in LTBI individuals to a 10% annual risk [2]. Conversely, Mtb infection itself "aggravates" the course of HIV-1 infection, increasing viral loads and acting as a predictor for worsening clinical disease course [3].

Current estimates are that one third of the world's population is infected with Mtb. Given that annual HIV-1 infection rates are increasing (http://apps.who.int/globalatlas/dataQuary/default.asp) [4], accurate diagnosis of both active and LTBI in HIV-1 positive individuals is of significant clinical value. Yet, to date, there is no gold standard for diagnosis of LTBI. Traditionally, the tuberculin skin test (TST) has been used to measure delayed hypersensitivity reaction following intra-dermal injection of purified protein derivative (PPD). However, the crude precipitate of Mtb culture supernatant (PPD) contains more than 200 proteins widely shared among mycobacteria, and consequently its use in the diagnosis of LTBI lacks both specificity and sensitivity [5, 6]. More recently, IFN-γ release assays (IGRA) such as QuantiFERON-TB Gold In-Tube and T-SPOT.TB have been developed [7]. IGRA improve the specificity of LTBI diagnosis by measuring cytokine production by immune cells to Mtb-specific proteins, transcribed from genes not found in BCG and most other environmental mycobacteria [7]. Early secreted antigenic target-6 and culture

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The detailed Materials and methods for Technical comments are available online in the Supporting information

filtrate-10 are the two best studied to date and can improve the sensitivity of LTBI diagnosis to 96% compared with 69% achieved by TST [7].

Despite the advantages of using IGRA over TST, questions have been raised as to the usefulness of *ex vivo* IFN-γ-based T-cell assays in LTBI diagnosis in HIV-1 seropositive individuals [8, 9]. HIV-1 infection is characterized by a reduction in the proliferative capacity and IFN-γ response of Mtb-specific $CD4^+$ T cells. This, when coupled with the overall decline in $CD4^+$ T-cell number and the poor concordance between different IGRA in immunocompromised individuals [8, 9], has raised concern as to the sensitivity of these assays in HIV-1 seropositive individuals [9–12], and prompted the Centre for Communicable Diseases (USA) to recommend caution in the use of IGRA when testing HIV-infected persons [13, 14].

To address these issues, we sought to further improve the sensitivity of conventional *ex vivo* T.Spot, TB assays by specific expansion of TB-specific T cells *in vitro*. Cultured IFN-γ ELISpot has been shown to increase the sensitivity of detection of low frequency and low magnitude T-cell responses compared with standard *ex vivo* assays in a number of disease settings [15–17]. The increased sensitivity is achieved by a 10-day, *in vitro*, peptide-stimulated culture, during which PBMC are supplemented with lymphoproliferative and anti-apoptotic cytokines (IL-2 and IL-7, respectively). Culture may enable expansion of antigen-specific T-cell populations that are both different from and additional to those detected by *ex vivo* assays to be measured [16].

Using a laboratory-modified version of the T-Sport. TB test, we initially screened 27 HIV-1 seronegative and 27 seropositive Malawian individuals for *ex vivo* Mtb-specific T-cell responses. In accordance with current World Health Organisation (WHO) predictions and previous literature reports [4, 18], we found 13/27 HIV-1-negative individuals had Mtb-specific T-cell responses (48.1%) (Fig. 1A). On the contrary, HIV-1-positive participants had a much lower frequency, with only 3/27 HIV-1 participants (11.1%), having detectable Mtb-specific T-cell responses (Fig. 1B). We next compared the *ex vivo* responses detected in a subset of these participants (26 HIV-1 negative and 22 HIV-1 positive) with those observed following the cultured IFN-γ ELISpot technique. Briefly, 12/26 HIV-1 seronegative individuals had positive Mtb-specific T-cell responses detected *ex vivo*. Following cultured ELISpot, the magnitude of Mtb-specific responses increased >20fold, and, consistent with the previous studies [13–15, 17] the frequency at which Mtb-specific T-cell responses were detected among HIV-1 seronegative individuals increased from 12/26 *ex vivo* to 17/26 (65%) following cultured assay (Fig. 2A). More strikingly, a greater than 6-fold increase in the detection of Mtb-specific T-cell responses was observed in HIV-1 seropositive individuals tested; 1/22 *ex vivo* to 7/22 following culture (Fig. 2B). Importantly, Mtb-specific T-cell responses were detected in subjects with CD4 counts of <200 cells/mm^3, suggesting that this technique overcomes some of the difficulties observed in applying IGRA to subjects with low CD4 counts [9, 12].

To examine whether the improved sensitivity resulted from nonspecific amplification or *in vitro* priming, cultured Mtb-specific T-cell responses were compared between a population in which Mtb infection is rare (UK – incidence 3%) and a population in which Mtb is endemic (Malawi – 68%) [4, 18]. Following cultured ELISpot 26/41 (63%) HIV-1 seronegative, Malawian participants produced Mtb-specific T-cell responses (Supporting Information Fig. 1A), whereas 1/14 (7%) UK participants tested positive (Supporting Information Fig. 1B). Notably, the background for these assays was low and specific expansion of Mtb T cells was not observed in the absence of peptide stimulation (data not shown). The TB-specific response rates observed by cultured ELISpot are consistent with

UK wide estimates of LTBI, suggesting that specificity was retained over the culture period of the assay.

To our knowledge, this is the first demonstration that cultured IFN-γ ELISpot can be used to improve the detection of Mtb-specific T-cell responses among HIV-1-positive individuals. Notably, cultured PBMC can also be used for more detailed studies such as T-cell-epitope mapping and phenotyping using flow cytometry (data not shown). Although we have shown the cultured assay to be a reliable, experimental tool that is standardized for research purposes, it is not validated clinically. Additional studies are now required to determine the clinical relevance of cultured ELISpot responses and the LTBI rates in other cohorts from both TB endemic and nonendemic regions. However, the low cell number requirements mean that this technique should prove a valuable tool in future research of Mtb-specific T-cell responses in both HIV-1-negative and - positive individuals.

In summary, these results confirm that current diagnostic techniques are underestimating latent Mtb infection rates and provide methodology for more accurate and detailed analysis of Mtb-specific responses in HIV-1/TB coinfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IGRA	IFN-γ release assays
LTBI	latently infected with TB
Mtb	*Mycobacterium tuberculosis*
PPD	purified protein derivative
TB	tuberculosis
TST	tuberculin skin test

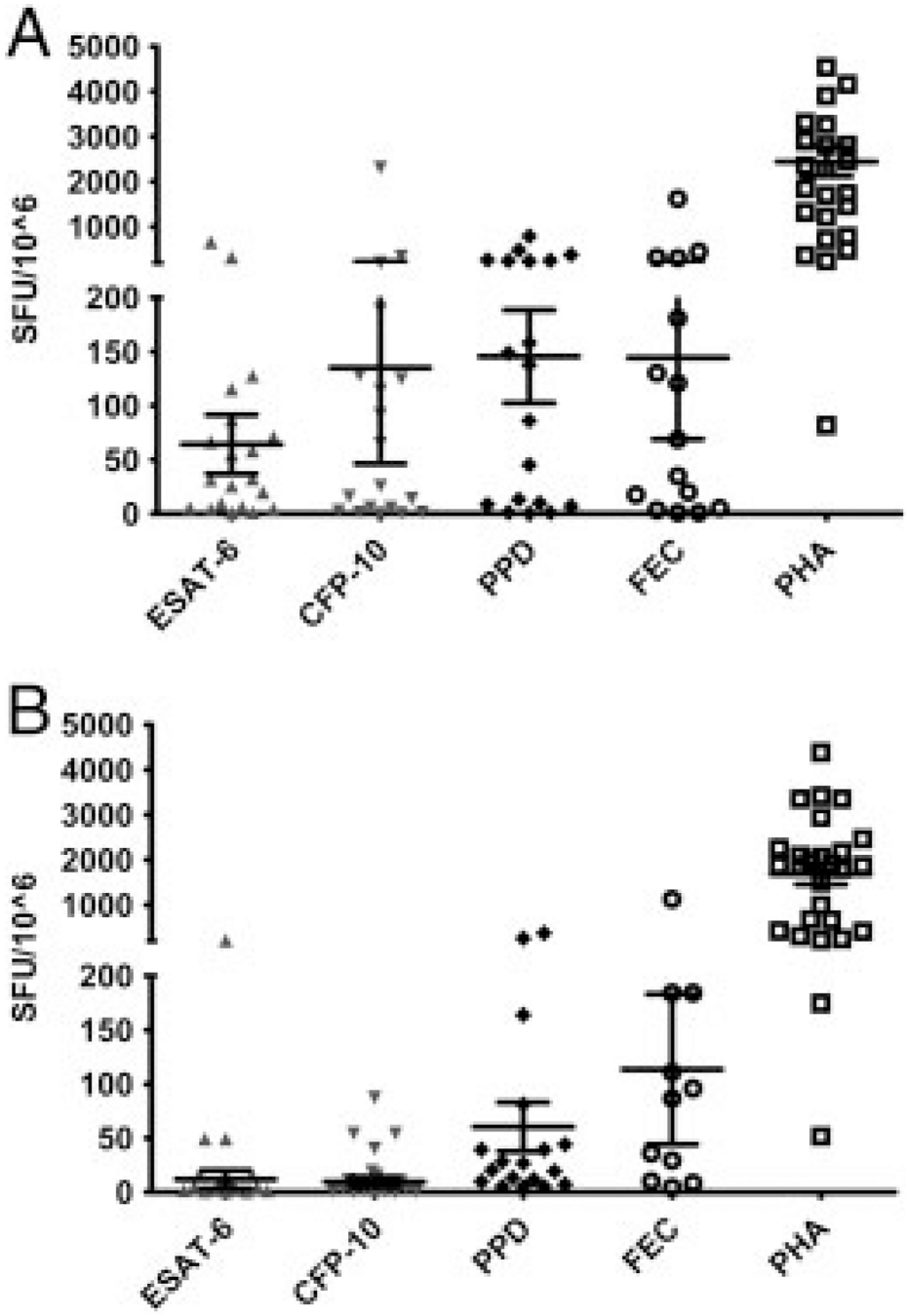

Figure 1.

Ex vivo IFN-γ ELISpot analysis of Mtb-specific responses in (A) 27 HIV-1 seronegative individuals and (B) 27 HIV-1-positive individuals from Malawi. Data presented as the number of spot forming units *per* million PBMC (SFU/10^6), following subtraction of mock-stimulated wells (background). All assays were performed in quadruplicate using Mtb-specific antigens (early secreted antigenic target-6 and culture filtrate-10), in addition to positive control antigens (PPD, FEC and PHA). Positive response defined as greater than or equal to 300 SFU/million PBMC, and or greater than 4× mock stimulation. Error bars represent standard error of mean.

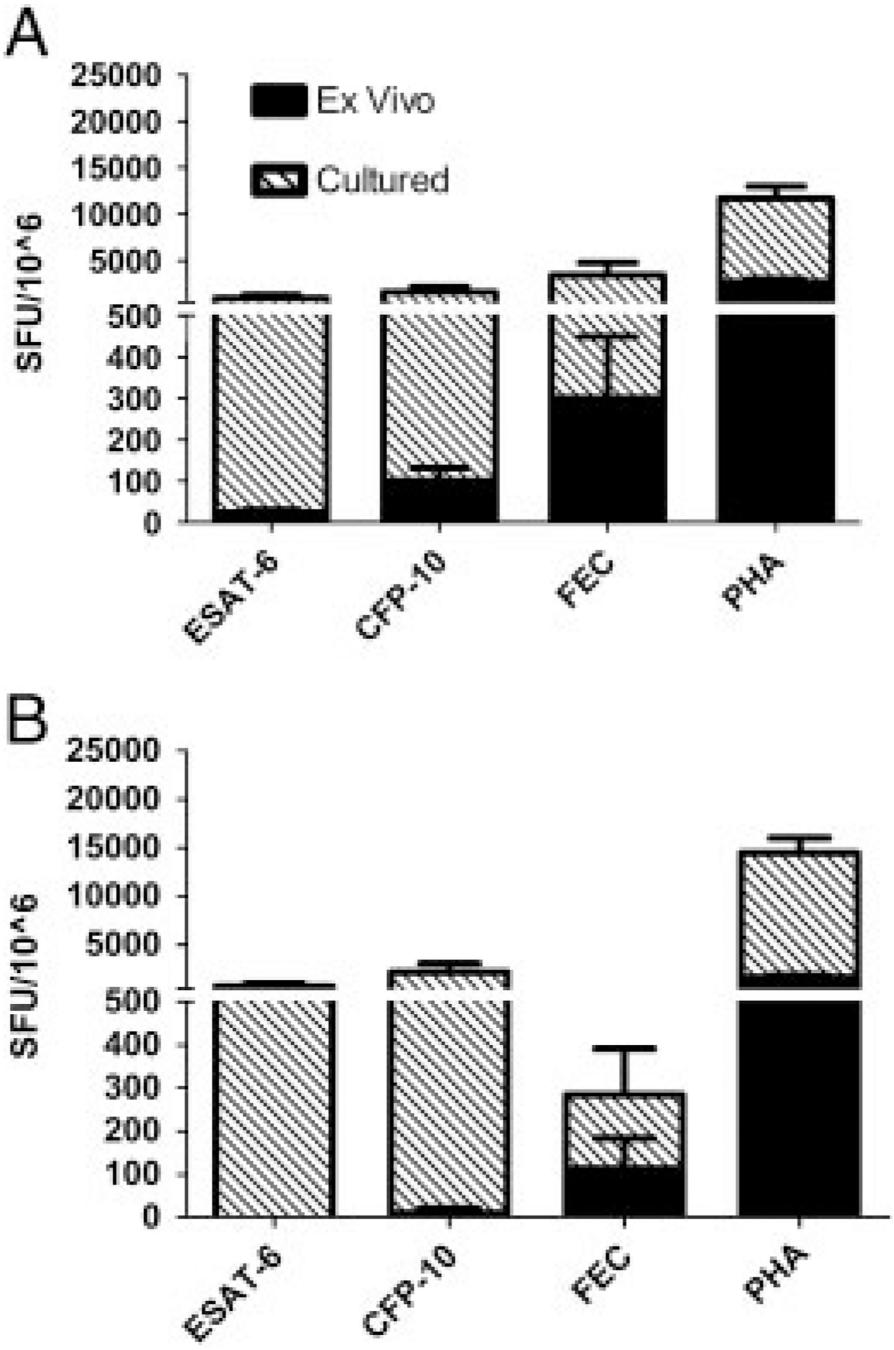

Figure 2.

Mtb-specific IFN-γ responses detected using *ex vivo* ELISpot analysis (solid bars) were compared with responses detected following cultured IFN-γ ELISpot (hatched bars) in (A) HIV-1-negative and (B) HIV-positive individuals. All data presented as average number of SFU *per* million PBMC following background subtraction. Criteria for a positive response were 4× mock-stimulated well and greater than 50 SFU/million PBMC (*ex vivo* assays) or 300 SFU/million PBMC (cultured assay). Error bars shown represent standard error of mean.